Suppression of Atherosclerosis in Cholesterol-Fed Rabbits by Diltiazem Injection

Masahiro Sugano, Yasuhide Nakashima, Toshio Matsushima, Kazuo Takahara, Masayuki Takasugi, Akio Kuroiwa, and Osamu Koide

The effects of diltiazem, a calcium antagonist, on the development of atherosclerosis were studied in Japanese white rabbits. The rabbits were examined at the end of 10 weeks on the following regimens; 1) a diet of standard pellets and daily intraperitoneal (ip) injections of saline; 2) a diet of pellets containing 1% cholesterol and daily ip injections of saline; or 3) a diet of pellets containing 1% cholesterol and daily ip injections of diltiazem (50 mg). The plasma total and LDL cholesterol levels for the third group were significantly lower than those of the cholesterol diet group. Macroscopically, atheromatous lesions covered 26.7% ± 6.7% (mean ± SE) of the intimal surface of the aorta in the second group, and 0.7% ± 0.3% in the third group (p < 0.005). The levels of cholesterol, calcium, and uronic acid in the aortic tissue of the second group were significantly higher than those in the third. We concluded that diltiazem administered intraperitoneally suppresses the plasma total and LDL cholesterol elevation induced by the cholesterol diet and inhibits experimentally-induced atherosclerosis. (Arteriosclerosis 6:237-241, March/April 1986)

Recently, Ca ++ antagonists, which appear to act by inhibiting transmembranous Ca ++ flux and/or the release and binding of intracellular Ca ++ flux and/or the release and binding of intracellular Ca ++ flux and/or the release and binding of intracellular Ca ++ flux, have been reported to suppress experimentally induced atherosclerosis.6-9 On the other hand, a few studies have demonstrated that nifedipine and diltiazem, given orally, have no antiatherosclerotic effect.10-12

Ca ++ antagonists are widely used to treat ischemic heart diseases, spastic angina, and hypertension. In this study we examined the effects of intraperitoneally administered diltiazem on plasma total cholesterol and cholesterol deposition in the aorta of rabbits given a 1% cholesterol diet.

Methods

Animals

Twenty male Japanese white rabbits, each weighing about 2.0 kg, were housed individually under controlled conditions and divided into three groups; 1) a standard diet group (n = 7) given 100 g of standard rabbit pellets (Clea Japan Incorporated), and receiving daily intraperitoneal (ip) injections of saline; 2) a cholesterol diet group (n = 8) fed pellets containing 1% cholesterol, and injected (ip) with saline; and 3) a diltiazem-treated group (n = 5) fed pellets containing 1% cholesterol and receiving daily ip injections of diltiazem (50 mg). Water was given ad libitum. After 10 weeks on these regimens, the rabbits were injected with 2 ml of a 5% pentobarbital solution and killed by bleeding from a femoral artery.

Diltiazem was generously supplied by the Tanabe Pharmaceutical Company (Tokyo, Japan).

Measurements of Plasma Constituents

At the beginning and end of the diet period, blood samples were drawn after an overnight fast, from a central ear artery of each rabbit into tubes containing Na2 EDTA (1 μg/100 μg). Plasma was analyzed for total protein, calcium, sodium, magnesium, and phosphorus with an automatic analyser (Hitachi Limited, Japan). For the determination of the lipoprotein fractions, the very low density lipoprotein (VLDL) fraction (d > 1.006) was separated by plasmapheresis13 from the bottom fraction containing the low density lipoprotein (LDL) and the high density lipoprotein (HDL) fractions. The HDL supernatant fraction of the LDL was obtained by addition of Dextran-Mg ++ to the bottom fraction.14 Cholesterol in the plasma, the bottom fraction, and the HDL fraction was measured directly, whereas the values for the VLDL and LDL fractions were calculated as the respective differences between the plasma, the bottom fraction, and the HDL fraction. Plasma triglycerides were also measured. The determinations of cholesterol and triglycerides were performed by enzymatic methods (Wako Pure Chemical Industries, Limited, Japan).
Macroscopic Examination and Chemical Analysis of the Aorta

Following exsanguination, the portion of the aorta from its origin at the aortic valve to the bottom iliac artery was isolated. The aorta was opened longitudinally along the midthoracic line, and the surface areas containing lesions were measured without staining by planimetry from an enlarged color photograph (×2 magnification). The atherosclerotic lesions were expressed as the percentage of the total aortic surface area. Four cross sections in each aorta were histologically examined: from the lower aortic arch, lower thoracic aorta, upper abdominal aorta, and lower abdominal aorta. Next, the aorta from each rabbit was cut into pieces (2×2 mm), were delipidated twice with Folch’s solution, and were stored in a dessicator at room temperature until the weight became constant.15 The lipid fractions thus obtained were used for the determination of cholesterol, free cholesterol, triglycerides, and phospholipids. The dried, defatted aortic tissues were used for the determinations of calcium, hydroxyproline, and uronic acid content. Calcium content was measured with an atomic absorption spectrophotometer (Model 606, Perkin-Elmer Corporation, Physical Electronics Division, Eden Prairie, Minnesota).16 Briefly, about 10 mg of aortic tissue was washed with 50% HNO3 and hydrogen-peroxide, and was dried. This material was dissolved in 2% HNO3 and used for the determination of calcium. About 5 mg of dried, defatted tissue was used for measurement of the hydroxyproline content by the method of Stegemann et al.17 For the determination of uronic acid, about 30 mg of tissue was digested by papain, was treated with trichloroacetic acid, and was dialyzed.18 Uronic acid was measured by the carbazole method.19 The contents of calcium, hydroxyproline, and uronic acid were expressed as milligram per gram of dried, defatted weight.

The difference between sequential mean values in the intragroup assay was evaluated by the paired-sample t test. Intergroup differences among the groups were evaluated by Student's t test.

Table 1. Body Weight and Plasma Constituents

<table>
<thead>
<tr>
<th></th>
<th>Standard diet (n = 7)</th>
<th>Cholesterol diet (n = 8)</th>
<th>Diltiazem-treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.35±0.16</td>
<td>3.18±0.12</td>
<td>2.90±0.26</td>
</tr>
<tr>
<td></td>
<td>(1.92±0.04)*</td>
<td>(1.98±0.04)*</td>
<td>(1.91±0.08)†</td>
</tr>
<tr>
<td>Calcium (Ca ++ ) (mEq/liter)</td>
<td>7.11±0.20</td>
<td>7.59±0.11c</td>
<td>7.12±0.14†</td>
</tr>
<tr>
<td></td>
<td>(7.94±0.11)§</td>
<td>(8.16±0.11)†</td>
<td></td>
</tr>
<tr>
<td>Sodium (Na +) (mEq/liter)</td>
<td>143.3±2.37</td>
<td>150.0±1.50d</td>
<td>158.6±1.33d</td>
</tr>
<tr>
<td></td>
<td>(144.9±0.48)§</td>
<td>(145.2±1.16)†</td>
<td></td>
</tr>
<tr>
<td>Potassium (K +) (mEq/liter)</td>
<td>4.30±0.40</td>
<td>4.63±0.19</td>
<td>4.12±0.39</td>
</tr>
<tr>
<td>Magnesium (Mg ++ ) (mEq/liter)</td>
<td>2.24±0.04</td>
<td>2.03±0.08b</td>
<td>2.08±0.13</td>
</tr>
<tr>
<td>Phosphorus (mEq/liter)</td>
<td>2.11±0.16</td>
<td>2.41±0.13</td>
<td>2.16±0.19</td>
</tr>
<tr>
<td></td>
<td>(3.34±0.28)§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>7.03±0.21</td>
<td>8.18±0.15a</td>
<td>6.78±0.22e</td>
</tr>
<tr>
<td></td>
<td>(6.43±0.21)§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values (means ± se) refer to measurements obtained at the end of each 10-week diet period. Values at the start of the study (indicated in parentheses) are given only if they differed from the subsequent values (*p < 0.001; †p < 0.005; ‡p < 0.01; §p < 0.05, paired t test). Statistical analysis among three groups was performed by Student's t test (*p < 0.001; ‡p < 0.005; †p < 0.02; compared to standard diet group. *p < 0.001; †p < 0.025; compared to cholesterol-diet group).

Results

Table 1 lists the body weights and biochemical analyses at the end of the feeding period. The body weight of the diltiazem-treated group was only slightly lower than that of the standard and cholesterol diet groups. However, total protein levels and plasma calcium and sodium concentrations in the diltiazem-treated group were significantly lower than those in the cholesterol diet group.

As shown in Figure 1, plasma triglyceride levels in the three groups were not altered during the feeding period. However, plasma total cholesterol and LDL cholesterol levels in the diltiazem-treated group at the 5th and 10th week were lower than those of the cholesterol diet group and the difference was significant at the end of the feeding period (10 weeks). VLDL cholesterol in the diltiazem-treated group was also lower than that in the cholesterol diet group at the 5th and 10th week of the feeding period, but the difference was not significant. By contrast, HDL cholesterol levels in the diltiazem-treated and cholesterol diet group at the 5th and 10th week of the feeding period were significantly higher than those in the standard diet group, but there was no difference between diltiazem-treated group and cholesterol diet group.

Macroscopically, various degrees of fibrous-fatty plaques were noticed on the aortic surface. Figure 2 shows the outlines of representative aortic plaques in each group. The plaques were small and with indefinite outlines in the standard diet group, extensive and with definite outlines in the cholesterol diet group, and small and scattered in the diltiazem-treated group.

Figure 3 shows that the percentage of lesions in the diltiazem-treated group was significantly lower than that in the cholesterol diet group, but similar to that in the standard diet group. Histological examination revealed two or three layers of intimal cells with rare lipid deposition on the intact internal elastica in the standard diet group. On the other hand, a marked increase of cellularity in the intima comprising fibroblasts, lipophages, and smooth muscle cells and an accumulation of collagen associated with deposition of lipid and of glycosaminoglycan in the intima and...
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subintimal media were noted in the aortic plaques of rabbits in the cholesterol diet group. Derangement and fragmentations of the internal elastic and subintimal medial elastic lamina were also demonstrated in the cholesterol diet group. The histologic findings of plaques of the aorta in the diltiazem-treated group were similar to those in the cholesterol diet group, but the changes were milder and fewer plaques had formed than in the cholesterol diet group.

The lipid components, calcium, hydroxyproline, and uronic acid of the aortic tissue in the three groups are shown in Tables 2 and 3. The cholesterol and phospholipid levels in the diltiazem-treated group were significantly lower than those in the cholesterol diet group. There were no differences in triglyceride or hydroxyproline content among the three groups. However, the calcium and uronic acid levels in the diltiazem-treated group were significantly lower than in the cholesterol diet group.

Figure 1. Changes of plasma total cholesterol (T-CHO), triglyceride (TG), and cholesterol in lipoprotein fractions. □ = standard diet group; ● = cholesterol diet group; ✦ = diltiazem group.

Figure 2. Tracing of representative aortic plaques in each group. Black areas represent fibrous-fatty aortic plaques.

Figure 3. Ratio of lesions to total surface area.
Discussion

Recently, several reports showed that Ca++ antagonists prevent lipid deposition in the aorta,8-9 while others indicated that Ca++ antagonists did not prevent the occurrence or progression of atherosclerosis.10-12 This discrepancy may be due to the different methods and materials used. The plasma cholesterol levels of the rabbits used in these studies were already high enough to initiate the atherosclerotic process20 before the start of the experiment. Although they used the same protocol as Henry et al.,7 Stender et al.10 found that nifedipine did not inhibit atherosclerosis and explained the discrepancy between their studies as due to the difference in the plasma cholesterol levels in the experimental and placebo animals during the feeding period, not the differences in rabbits used. They also suggested that the mechanism of the antiatherogenic action of verapamil and diltiazem may be associated with cellular function, which is much more affected by these drugs than by nifedipine because of the marked differences in the clinical electrophysiologic property from those of verapamil and diltiazem.21, 22

In this study, diltiazem, administered intraperitoneally, had an antiatherosclerotic effect by reducing plasma cholesterol and the aortic calcium and uronic acid levels in rabbits on a 1% cholesterol diet. The plasma total cholesterol, VLDL, and LDL cholesterol levels of the diltiazem-treated group were markedly reduced compared to those of the cholesterol diet group. Recently, Ohta et al.23 reported that nicardipine reduces plasma total cholesterol and LDL cholesterol levels in rats on a high cholesterol diet and suggested that these effects are due to potentiation of the transport of HDL from the blood to the liver, rather than to the inhibition of synthesis or the impairment of absorption from the intestine. However, Naito et al.12 and Ginsburg et al.9 reported that orally administered diltiazem did not lower plasma cholesterol in rabbits on a high cholesterol diet. This may be due to the difference in the method of administration of diltiazem.

We administered diltiazem intraperitoneally to avoid the individual variation in the absorption ratio by oral administration, because the metabolic pathway of drugs administered in both routes were the same. Briefly, diltiazem administered by oral and intraperitoneal routes enters the liver via the portal vein and has a first-pass effect. However, in a hyperlipidemic state, the lymph circulation was increased and entered the systemic circulation directly when the diltiazem was given orally, which might diminish the first-pass effect in liver.

Another possibility is the difference of the effective dose in the circulatory system. We used about 20 mg/kg of diltiazem intraperitoneally. The pharmacological effect of this amount on hemodynamics would correspond to 400 mg/kg of orally administered diltiazem.24 The oral dose of diltiazem in the studies by Naito et al. and Ginsburg et al. was about 100 mg/kg, about three times the clinical dose used for humans. Further studies on this point are needed.

The levels of cholesterol, calcium, and uronic acid in the aortic tissue of the diltiazem-treated group were significantly lower than that in the cholesterol diet group. Macroscopic and histological examinations suggested that diltiazem suppresses the atherosclerotic changes in rabbits on a high cholesterol diet. Our results are in agreement with those reported by others.8-9

Hypertension is a well-defined potent risk factor in atherosclerosis. In this study, diltiazem induced a 30% decrease in blood pressure, which was well tolerated. This reduction may have played a role in the observed antiatherosclerotic effect, although the effect of reduced blood pressure by the hypotensive agents such as β-blocker and hydralazine on experimental atherosclerosis has been reported to be negligible.25, 26 Kramsch et al.6 described the possible mechanisms of Ca++ antagonists in the inhibition of atherogenesis as involving: changes in platelet aggregation, the effects on smooth muscle cells, vascular endothelial cell contraction leading to increases in permeability, and the regulation of intracellular messengers for protein or some enzymes or both. These effects are probably due to a "final common pathway" of the Ca++ antagonist, the inhibition of calcium influx into cells, and the reduction of the amount of calcium subsequently bound to cytoplasmic macromolecules. Although it has not yet been established that arterial Ca++ deposition is related to the progression of atherosclerotic disease, current evidence suggests that calcium metabolism in arterial tissue plays a pathogenic role in atherosclerosis.27-34 The calcium antagonist affecting the calcium metabolism may be related to the atherosclerosis.

We mainly studied the effects of diltiazem on atherosclerosis, and not the mechanism of its action. Thus, further studies on the effects of Ca++ antagonists on atherogenesis are needed.

Table 2. Lipid Contents of Aorta

<table>
<thead>
<tr>
<th></th>
<th>Standard Diet (n = 7)</th>
<th>Cholesterol Diet (n = 8)</th>
<th>Diltiazem-treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/g dw)</td>
<td>5.2 ± 0.4</td>
<td>29.4 ± 9.2†</td>
<td>8.8 ± 3.6‡</td>
</tr>
<tr>
<td>Esterified cholesterol (%)</td>
<td>8.7 ± 5.2</td>
<td>39.7 ± 19.4¢</td>
<td>25.1 ± 11.6§</td>
</tr>
<tr>
<td>Phospholipid (mg/g dw)</td>
<td>15.5 ± 3.2</td>
<td>29.5 ± 8.4‡</td>
<td>15.5 ± 3.2†</td>
</tr>
<tr>
<td>Triglyceride (mg/g dw)</td>
<td>40.5 ± 15.2</td>
<td>56.3 ± 25.3</td>
<td>40.1 ± 18.2</td>
</tr>
</tbody>
</table>

Values are means ± se of measurements obtained at the end of each 10-week diet period. Esterified cholesterol was calculated as the differences between total cholesterol and free cholesterol and is represented as the percentage of total cholesterol. dw = dry weight. Statistical analyses were performed by Student's t test (*p < 0.001; †p < 0.005; ‡p < 0.01; §p < 0.05; compared to standard diet group; †p < 0.01; †p < 0.05; compared to cholesterol diet group).

Table 3. Calcium, Uronic Acid, and Hydroxyproline Contents of Aorta Tissue

<table>
<thead>
<tr>
<th></th>
<th>Standard Diet (n = 7)</th>
<th>Cholesterol Diet (n = 8)</th>
<th>Diltiazem-treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/g dw)</td>
<td>1.83 ± 0.21</td>
<td>3.53 ± 0.32*</td>
<td>1.63 ± 0.13†</td>
</tr>
<tr>
<td>Uronic acid (mg/g dw)</td>
<td>3.43 ± 0.16</td>
<td>3.70 ± 0.11</td>
<td>2.51 ± 0.13†</td>
</tr>
<tr>
<td>Hydroxyproline (mg/g dw)</td>
<td>32.94 ± 1.68</td>
<td>32.91 ± 2.08</td>
<td>38.38 ± 1.83</td>
</tr>
</tbody>
</table>

Values are represented as means ± se; dw = dry weight. Statistical analyses were performed by Student's t test (*p < 0.001, compared to standard diet group; †p < 0.001, compared to 1% cholesterol diet group).
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References


Index Terms: diltiazem • aorta • cholesterol • diet • triglycerides • plasma total cholesterol • low density lipoprotein cholesterol • calcium antagonist • uronic acid • plaque • hydroxyproline
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